

# THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office

November 15, 2004

THIS IS TO CERTIFY THAT ANNEXED HERETO IS A TRUE COPY FROM THE RECORDS OF THE UNITED STATES PATENT AND TRADEMARK OFFICE OF THOSE PAPERS OF THE BELOW IDENTIFIED PATENT APPLICATION THAT MET THE REQUIREMENTS TO BE GRANTED A FILING DATE.

APPLICATION NUMBER: 60/464,456  
FILING DATE: April 22, 2003  
RELATED PCT APPLICATION NUMBER: PCT/US04/12441

BEST AVAILABLE COPY

Certified by

Jon W Dudas

Acting Under Secretary of Commerce  
for Intellectual Property  
and Acting Director of the U.S.  
Patent and Trademark Office





1133 U.S. PTO

04-23-031464456.042204/PROV

Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No.

EV 119099526 US

1133 U.S. PTO



04/22/03

INVENTOR(S)					
Given Name (first and middle (if any))		Family Name or Surname		Residence (City and either State or Foreign Country)	
Mark		Denison		Nashville, Tennessee	
<input type="checkbox"/> Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
Live Attenuated Nidovirus Vaccines					
Direct all correspondence to: CORRESPONDENCE ADDRESS					
<input checked="" type="checkbox"/> Customer Number		32425			
OR		Type Customer Number here			
<input type="checkbox"/> Firm or Individual Name		PATENT TRADEMARK OFFICE			
Address					
Address					
City		State		ZIP	
Country		Telephone		Fax	
ENCLOSED APPLICATION PARTS (check all that apply)					
<input checked="" type="checkbox"/> Specification		Number of Pages		42	
<input checked="" type="checkbox"/> Drawing(s)		Number of Sheets		9	
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76		<input type="checkbox"/> CD(s), Number			
		<input type="checkbox"/> Other (specify)			
METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT					
<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.				FILING FEE AMOUNT (\$)	
<input checked="" type="checkbox"/> A check or money order is enclosed to cover the filing fees					
<input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge filing fees or credit any overpayment to Deposit Account Number:		50-1212		\$80.00	
<input type="checkbox"/> Payment by credit card. Form PTO-2038 is attached.					
The invention was made by an agency of the United States Government or under a contract with an agency of the United States Government.					
<input type="checkbox"/> No.					
<input checked="" type="checkbox"/> Yes, the name of the U.S. Government agency and the Government contract number are: NIH/NIAID 5R01 A126603-15					

Respectfully submitted,

SIGNATURE

TYPED or PRINTED NAME

Steven L. Highlander

TELEPHONE

(512) 536-3184

Date

04/22/2003

REGISTRATION NO.

(if appropriate)

Docket Number:

37,642

VBLT:038USP1

## USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

This collection of information is required by 37 CFR 1.51. The information is used by the public to file (and by the PTO to process) a provisional application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 8 hours to complete, including gathering, preparing, and submitting the complete provisional application to the PTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, Washington, D.C. 20231. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Box Provisional Application, Assistant Commissioner for Patents, Washington, D.C. 20231.

60464456 .042203

**PATENT**

**ATTY DKT: VBLT:038USP1**

**PROVISIONAL APPLICATION FOR UNITED STATES LETTERS PATENT**

**for**

**LIVE ATTENUATED NIDOVIRUS VACCINES**

**by**

**Mark Denison**

EXPRESS MAIL NO.: EV 119099526

DATE OF DEPOSIT: April 22, 2003

## **BACKGROUND OF THE INVENTION**

The government owns rights in the present invention pursuant to grant number SRO1 A126603-15 of the National Institutes of Health and National Institute Allergy Infectious Disease.

5

### **1. Field of the Invention**

The present invention relates generally to the fields of microbiology, immunology and virology. More particularly, it concerns live, attenuated Nidovirus vaccines and methods for preventing or limiting Nidovirus infections.

### **10 2. Description of Related Art**

Coronaviruses have been long known to cause important diseases in a wide variety of animal species, including humans, cattle, swine, chickens, dogs, cats and mice. Coronavirus diseases in non-human species may be quite severe, and devastating in domestic livestock such as pigs, cattle and chickens. The characterized human coronaviruses - HCoV-229E and HCoV  
15 OC43 - are significant causes of upper respiratory infections, responsible for 10-35% of human colds. Studies of human coronaviruses have been limited by their lack of growth in culture from primary isolates, and by the lack, until recently, of reverse genetic approaches for their study. Thus, while the human coronaviruses are arguably two of the most economically important viruses in humans, ongoing research has been pursued only by a handful of dedicated  
20 investigators.

The emergence of a new human coronavirus associated with "severe acute respiratory syndrome" (SARS) surprised many scientists and public health officials, but has highlighted characteristics of coronaviruses well known to investigators. The coronaviruses have high rates of mutagenesis and homologous RNA recombination. In fact, template switching and  
25 recombination are essential to the normal life cycle of the viruses. In addition, the species barrier for coronaviruses has been predicted to be tenuous. Studies of coronaviruses in culture have demonstrated the ability of coronaviruses to adapt for replication in cells of different species. In addition, some studies have demonstrated that the murine coronaviruses may cause disease in primates following direct inoculation into brain. Finally, coronaviruses have been  
30 proposed, based on evolutionary studies, to have acquired genes from other viruses or cells,

probably by recombination events. The emergence of a new coronavirus pathogenic for humans, by either adaptation of an animal virus, or by recombination of two coronaviruses during a coinfection, is consistent with these features of coronavirus evolution, replication and maintenance in populations.

5 Vaccine approaches for important domestic animal coronavirus diseases, specifically the chicken avian infectious bronchitis virus (IBV), porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCV), bovine coronavirus (BCV) and feline infectious peritonitis virus (FIPV), have been developed or attempted over the past 20 years. The approaches to vaccine development have been based on non-targeted natural attenuation, virus expression  
10 vectors, virus inactivation, recombinant viral structural proteins, and novel approaches to deliver or adjuvant vaccines. Responses and protectivity of these vaccines have varied widely, but no vaccine has been shown to possess all of the characteristics of safety, stability and efficacy.

For FIPV, live-attenuated vaccines, inactivated virus vaccines, and subunit vaccines based on recombinant or purified spike protein, have not only failed to protect against FIPV  
15 disease, but in fact have resulted in immune enhancement of infection and disease, a response disturbingly reminiscent of the result following vaccination humans with inactivated vaccines for measles and respiratory syncytial virus. The most useful animal coronavirus vaccine has been the live-attenuated vaccine for IBV. However, its efficacy is still clearly less than optimal. In addition, reversion to virulence may occur, and recombination of the vaccine strain with wild-  
20 type viruses has occurred, with disease in chickens caused by the recombinant vaccine-wild-type viruses.

For the most part, vaccines have not been pursued in the past for human coronaviruses, likely because the frequency and severity of infections could not be well defined, and the determinants for protection have not been identified. It is also known that 229E and OC43 can  
25 reinfect humans, possibly as often as every other year, suggesting that vaccine strategies may need to be targeted toward limitation of disease severity, since prevention may not be possible.

Together, the known biological properties of coronaviruses, as well as the concerns with limited protection or immune enhancement of disease by coronavirus vaccines, are compelling arguments for a new approach in the development of live, attenuated vaccines that are less  
30 subject to reversion and recombination, but possess normal pathways for infection and immune response. This need is all the more critical in light of the emerging human SARS situation.

### SUMMARY OF THE INVENTION

Thus, in accordance with the present invention, there is provided a live, attenuated virus of the order *Nidovirales*, said virus characterized as comprising a genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage. The virus may be of the family *Coronaviridae*, such as a coronavirus or a torovirus. Particular coronaviruses are avian infectious bronchitis virus, bovine coronavirus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E, human coronavirus OC43, murine hepatitis virus, porcine epidemic diarrhea virus, porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, rat coronavirus, turkey coronavirus, severe acute respiratory syndrome virus, or rabbit coronavirus. Toroviruses include Berne virus or Breda virus. The virus may also be of the family *Arteriviridae*, such as arteriviruses like equine arteritis virus, Lelystad virus or simian hemorrhagic fever virus.

The cleavage site may be a C1-C14 cleavage site. In particular, the cleavage site may be a murine hepatitis virus p28-p65 or p65-p210 cleavage site. The cleavage site may contain an amino acid deletion, an amino acid insertion or an amino acid substitution. Alternatively, cleavage site may be wild-type, but cleavage is reduced or eliminated by an allosteric mutation in the proteinase responsible for cleavage. The replicase polyprotein may comprise at least a second proteinase cleavage site that exhibits reduced as compared to wild-type, or no cleavage.

In another embodiment, there is provided a method of inducing an anti-viral immune response in a host comprising administering to said host a live, attenuated vaccine of the order *Nidovirales*, said vaccine characterized as comprising a genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage. The vaccine may be of the family *Coronaviridae*, such as a coronavirus or a torovirus. Particular coronaviruses are avian infectious bronchitis virus, bovine coronavirus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E, human coronavirus OC43, murine hepatitis virus, porcine epidemic diarrhea virus, porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, rat coronavirus, turkey coronavirus, severe acute respiratory syndrome virus, or rabbit coronavirus. Toroviruses include Berne virus or Breda virus. The vaccine may also be of the family *Arteriviridae*, such as arteriviruses like equine arteritis virus, Lelystad virus or simian hemorrhagic fever virus.

The cleavage site may be selected from the C1, C2, C3, C4, C5, C6, C7, C8, C9, 10, C11, C12, C13 and C14 cleavage sites, with C1 representing the first recognized cleavage site in the polyprotein. In particular, the cleavage site may be a murine hepatitis virus p28-p65 or p65-p210 cleavage site. The cleavage site may contain an amino acid deletion, an amino acid insertion or  
5 an amino acid substitution. Alternatively, cleavage site may be wild-type, but cleavage is reduced or eliminated by an allosteric mutation. The replicase polyprotein may comprise at least a second proteinase cleavage site that exhibits reduced as compared to wild-type, or no cleavage. The vaccine may be administered (would change to intramuscularly, subcutaneously, intradermally, intranasally, or orally). The method may further comprise administering an  
10 immunostimulant such as an adjuvant like alum, MF-59, QS-21, or others, or a biologic immunomodulatory molecule such as a cytokine, immunological receptor, or antibody. The host may be a dog, a cow, a pig, a cat, a mouse, a rat, a horse, a chicken, a turkey, a monkey or a human.

In yet another embodiment, there is provided a nidovirus genome, said genome encoding  
15 a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage. Also provided is a nidovirus replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage.

In still yet another embodiment, there is provided a vaccine comprising (a) a live, attenuated virus of the order *Nidovirales*, said virus characterized as comprising a genome  
20 encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage, and (b) a pharmaceutically acceptable diluent. The vaccine may be formulated as a unit dose of  $10^6$  to  $10^{14}$  infectious particles, and may be formulated to be dispensed as unit doses of 0.1 ml to 1.0 ml. The vaccine may further comprise a preservative. The vaccine may be lyophilized.

## **BRIEF DESCRIPTION OF THE DRAWINGS**

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

**FIG. 1 - MHV-A59 genome organization and proteins.** The MHV-A59 genome is 32 kB in length. Gene 1 (replicase gene) is translated from input genome RNA to yield the ORF 1a polyprotein or the ORF 1a/1b polyprotein. Genes 2 through 7 are expressed from a 3' nested set of subgenomic mRNAs. Confirmed or predicted mature replicase gene products are shown as rectangles in gene 1. Other gene 1 proteins are labeled by either mass in kD or by the known or putative function; PLP1 and PLP2 – papain-like proteinase 1 and 2; 3C – 3C-like proteinase; pol – putative RNA dependent RNA polymerase; hel – NTPase/helicase. The structural proteins M and N are encoded by genes 6 and 7 respectively. Beneath the schematic is shown a relative pattern of processing of the amino terminal p28, p65 and p210 proteins. P28 is detected first, with p65 and p210 detected with similar timing. Arrows show elongating polyprotein with relative size required for processing to occur.

**FIG. 2 - Schematic of the N- terminal 3300 amino acids of the MHV replicase polyprotein.** The size and organization of p28, p65 and p210 protein domains are shown as rectangles. P210 is shown in grey. The cleavage sites are shown; CS1, CS2 and CS3, along with cleavage site dipeptides indicated. The p210 enlargement shows the known and putative domains within p210; Ac (acidic domain), papain-like proteinase 1 (PLP1), X domain, papain-like proteinase 2 (PLP2), and Y domain. The location of zinc finger motifs (Z1 and Z2) are shown as dark bars in PLP1 and PLP2. Predicted membrane spanning sequences are shown in the Y domain as dark vertical lines.

**FIG. 3 - Comparison of N-terminal regions of groups 2, 1 and 3 coronaviruses and PLP activity.** The N-terminal regions of the replicase polyproteins from MHV (and BCV), HCoV-229E, and IBV are shown, with the p210/p195 proteins indicated by grey boxes. Locations of respective cleavage sites CS1, CS2, and CS3 are indicated. Arrows with solid lines indicate confirmed processing by PLP1 and PLP2 from in vitro studies. Arrows with dashed

lines indicate predicted cleavage activities. The black box at IBV PLP1 indicates a poorly conserved domain without catalytic residues or activity (modified from Ziebuhr *et al.*, 1999).

**FIG. 4 - Mutagenesis of CS1 residues and generation of virus mutants.** Nucleotide and amino acid mutations from sequenced mutant viruses. \**In vitro* cleavage after Baker, (1993) and Hughes *et al.* (1995).

**FIG. 5 - Single cycle growth of wild-type and CS1 mutant viruses.** DBT cells were infected at an MOI of 10 pfu/ml and samples obtained for plaque assay on DBT cells at the times indicated. The viruses are indicated in the legend. wt-wild-type lab MHV-A59; ic wt- assembled wild-type A59; numbers (8,9,3,4,5) indicate icMut 8,9,3,4, and 5. \*\*Mut 8 12 h time point is included, even though only one replicate was possible.

**FIGS. 6A-B - Protein processing at CS1.** (FIG. 6A) DBT cells were infected with viruses as indicated above the lanes. At 5.5 h p.i. cells were radiolabeled with [35S]met for 90 min. Post nuclear lysates re-immunoprecipitated with UP102 (ap28,ap65). Markers are to the left of gel. P28, p65, and new p93 protein are indicated by arrows. (FIG. 6B) Lysates from ic Mut 9 (ic-9) and icMut 5 (ic-5) infected cells (I) and mock infected cells (M) were immunoprecipitated with ap28 antibodies or preimmune serum from the same rabbit. Note light band at same mobility as p28 in infected-preimmune IP and mock-infected immune IP.

**FIGS. 7A-C - Intracellular localization of viral proteins in DBT cells infected with MHV-A59 or ic Mut 5.** DBT cells on glass coverslips were infected with virus at an MOI=1 for 6 h, fixed, and probed with antibodies against MHV proteins. Images were obtained on a Zeiss LSM 510 confocal microscope. (FIG. 7A) MHV-A59-infected cells probed with a-p28 (red) and a-p65 (green); (FIG. 7B) MHV-A59-infected cells probed with a-p28 (red) and a-N (green); (FIG. 7C) icMut5-infected cells probed with a-p65 (green) and a-N (red). Co-localization is shown as yellow pixels in all images.

**FIG. 8 - Alignment of gene 1 polyprotein products of SARS (TOR2 strain) and MHV (strain A59).** The replicase polyproteins of SARS and MHV exhibit considerable conservation of organization. Predicted or confirmed mature protein products are represented as white boxes. Mature proteins are named according to their predicted molecular mass (*e.g.*, p25, p65, *etc.*) or by putative or confirmed function (MP1; membrane protein 1, 3CPro; 3 C-like proteinase, MP2; membrane protein 2, pol; RNA-dependent RNA polymerase, hel; RNA helicase). Gray shaded areas represent functional domains of the papain-like proteinases 1 and 2

(PLP-1 and PLP-2). The catalytic histidine and cysteine residues of the putative PLP-1 for SARS are denoted by vertical lines. Amino-acid residue numbers are represented by the black bar at the bottom of the figure.

5 **FIG. 9 - Cleavage site mutations of CS1 and CS2.** Proposed mutations are shown that allowed cleavage or blocked cleavage *in vitro*. The horizontal bar indicates deletion. \*Indicates mutations already engineered in fragment A and \*\* indicates mutations recovered from viable virus mutants. Vertical arrow indicates cleavage site. (Based on refs. 1-3).

## **DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS**

### 10 **I. The Present Invention**

The present inventors have used the murine coronavirus, mouse hepatitis virus (MHV), to demonstrate the feasibility of developing attenuated Nidovirus vaccines. MHV is a well-developed model for understanding the replication, pathogenesis, and immune determinants of coronavirus infections and disease. In addition, MHV has been an important virus model for  
15 demyelinating encephalitides, specifically multiple sclerosis. In this study, the inventors used MHV to determine if mutations in the coronavirus replicase gene could yield stably attenuated mutants.

Specifically, the inventors initially tested the hypothesis that the first cleavage event in the MHV replicase polyprotein, between p28 and p65, was required for viral replication.  
20 Surprisingly, though the mutants showed greatly reduced virus replication, they were still replicative. This result led to the concept of using such mutants as stably attenuated vaccines with distinct advantages. First, no proteins corresponding to the replicase proteins have been identified in virions, nor do they appear to be specifically targeted to the cell surface. Thus, there would be expected to be less immune pressure for reversion during infection. Second, other  
25 deletions or alterations in the proteins themselves appear to be lethal for replication, so that it will be more difficult for the virus to introduce second site compensating mutations. And third, cleavage knockout mutants that delete P2, P1 and P1' still permit recovery of viable virus. Thus, the tolerance for cleavage site deletion can be exploited to create virus with even less likelihood of reversion to virulence.

Clearly, the primary concern in using attenuated vaccines is the possibility of wild-type reversions. And it certainly has been well demonstrated that coronaviruses have a variety of mechanisms, as well as the capacity, to recover from seemingly insurmountable impairments such as dramatic rearrangements and deletions of genes downstream from the replicase. The high rates of mutation and homologous recombination all provide mechanisms to recover virulence. However, in absence of recombination during coinfection, the likelihood of regeneration of a cleavage site deletion would be very unlikely, particularly since the replication defect, while significant, is not lethal. Further, the results reported here suggest the possibility that the virus may tolerate abolition of other replicase polyprotein cleavages.

For human viruses acquired by the respiratory route, live-attenuated vaccines impaired in protein processing would have several potential advantages. Because there is no alteration in the viral structural glycoproteins, it is predicted that replicase protein cleavage mutants would have normal "wild-type" transmission, tropism, attachment, entry and uncoating, and thus could theoretically be administered by oral, intranasal or inhaled approaches. The initial replication and spread from the respiratory epithelium and lymphoid organs might allow for the development of both systemic and mucosal immunity.

Studies with other animal coronavirus vaccines suggest that viral replication is necessary for protection from virus challenges. The use of a virus stably attenuated in replication also avoids concerns about atypical infections with wild-type viruses following vaccination with inactivated viruses or purified viral proteins, such as occurred with measles virus and respiratory syncytial virus, and also seen with the vaccines for the feline coronavirus, FIPV. Most importantly, the use of a live-attenuated virus allows for both humoral and cellular immunity.

Thus, as a general strategy for nidovirus vaccines, the inhibition of polyprotein processing is widely applicable to viruses with significantly different hosts, virulence, pathogenesis and disease. All known nidoviruses use a similar strategy to translate and process their replicase proteins from a polyprotein. The similarities in the viral proteinases, proteins and cleavage sites, particularly in the group 2 coronaviruses, indicate that similar strategies could be used to develop other attenuated virus vaccine strains. Importantly, in the context of the newly emerged human SARS coronavirus, the corresponding cleavage sites may be rapidly identified, targeted for mutations, and quickly tested for stable attenuation.

## II. Order *Nidovirales*

Nidoviruses are positive-stranded RNA viruses infect a wide range of vertebrates. The virions are enveloped, pleomorphic, spherical, or kidney-shaped. Surface projections of envelope distinct; club-shaped; dispersed evenly over all the surface. Two families are established: Family *Arteriviridae* and Family *Coronaviridae*.

### A. *Coronaviridae*

This virus infects host in the Domain Eucarya, Kingdom Animalia, Phylum Chordata, Subphylum Vertebrata, Classes Mammalia and Aves, Orders Primates, Carnivora, Perissodactyla, Artiodactyla, Rodentia, and Lagomorpha. It is transmitted by means not involving a vector. World-wide distribution is likely.

Virions are enveloped, slightly pleomorphic, spherical or kidney shaped, and about 120-160 nm in diameter. Surface projections of envelope are distinct, club-shaped, spaced widely apart and dispersed evenly over all the surface. Nucleocapsids are rod-shaped (straight or bent), about 9-13 nm in diameter. Virions associated RNA nucleocapsids exhibit helical or tubular symmetry.

Molecular mass (Mr) of the virion  $400 \times 10^6$ . Buoyant density is 1.23-1.24 g cm<sup>-3</sup> in CsCl, and 1.15-1.19 g cm<sup>-3</sup> in sucrose. The sedimentation coefficient is 300-500S. Under *in vitro* conditions, virions are stable in acid environment (pH 3), relatively stable in presence of Mg<sup>++</sup>. Virions are sensitive to heat, lipid solvents, non-ionic detergents, formaldehyde, and oxidizing agents.

Virions contain one molecule of linear positive-sense single stranded RNA with a total genome length is 20,000-33,000 nt. The 5' end of the genome has a cap, and the 3' end has a poly(A) tract. Subgenomic mRNA is found in infected cells.

Five structural virion proteins found ranging in size between 18,000 and 220,000 Da. The first is the surface glycoprotein or spike (S) protein. The S protein is responsible for attachment to cells, hemagglutination and membrane fusion. It has a carboxy-terminal half with a coiled-coil structure. The second largest protein (30,000-35,000 Da) is the integral membrane protein (M) which spans the virus envelope three times, with only 10% protruding at the virion surface. The third largest protein (50,000-60,000 Da) is the nucleocapsid protein (N). The fourth largest protein (65,000 Da) is the hemagglutinine-esterase protein (HE), which forms short

surface projections, and can have receptor binding, hemagglutination and receptor destroying activities. The fifth largest protein (10,000-12,000 Da) is tentatively designated as the small membrane protein (sM), detected in avian infectious bronchitis virus (IBV) and porcine transmissible gastroenteritis virus (TGEV).

5 The virus exhibits distinct antigen determinants on envelope and spikes, those corresponding to each of the major structural glycoproteins - S, HE, M, and N. Antigenic specificity of virion can be determined by neutralization tests (S and HE), or complement fixation tests (M). Protective immunity is induced in form of complement independent neutralizing antibodies.

10 The *Coronaviridae* family is split into two groups - coronavirus and torovirus. Coronaviruses include avian infectious bronchitis virus, bovine coronavirus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E, human coronavirus OC43, murine hepatitis virus, porcine epidemic diarrhea virus, porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, rat coronavirus, turkey coronavirus, severe  
15 acute respiratory syndrome virus, rabbit coronavirus, or the recently identified SARS associated human coronavirus. Toroviruses include Berne virus or Breda virus.

#### B. *Arterivirus*

The family *Arterivirus* infects hosts of the Domain Eucarya, Kingdom Animalia, Phylum  
20 Chordata, Subphylum Vertebrata, Class Mammalia, Orders Primates, Perissodactyla, Artiodactyla, and Rodentia, and is transmitted by means not involving a vector. Serological relationships between different members are not detectable, *i.e.*, cross-reactivity not found.

Virions are enveloped, spherical, and about 60 nm in diameter. Surface projections on envelope not present, but honeycomb-like subunits of 10-14 nm have been observed.  
25 Nucleocapsids are isometric and about 35 nm in diameter. The virion RNA-nucleocapsid exhibits icosahedral symmetry. Surface projections on nucleocapsid are small dispersed over the entire surface.

Buoyant density is 1.17-1.2 g cm<sup>-3</sup> in CsCl, and 1.13-1.17 g cm<sup>-3</sup> in sucrose. The sedimentation coefficient 200-230S.

30 Virions contain one molecule of linear single stranded RNA. The total genome length is 13000 nt. The 5' end of the genome has a cap (cap sequence m<sup>7</sup>G5ppp5'GmpNp). The 3' end has

a poly(A) tract that is about 50 nucleotides long. Each virion contains longer than full length copy.

At least four structural virion proteins are found. Protein sizes range from 30,000 to 42,000 Da. The first is an N-glycosylated surface protein designated GL, which is about 25,000 Da. The second is an N-glycosylated surface protein designated GD, which is about 16,000 Da. The third is a non-glycosylated triple membrane spanning integral membrane protein designated M. The fourth is a 12,000 Da nucleocapsid protein, designated N, also non-glycosylated. Virions contain 10 % lipid (envelope).

Selected Arteriviruses include equine arteritis virus, Lelystad virus and simian hemorrhagic fever virus.

### III. Replicase Polyprotein

#### A. Gene and Protein Structure

The coronavirus replicase gene (also known as gene 1 or the polymerase gene) comprises 22 kB of the coronavirus genome, corresponding to some 7800 amino acids, and is composed of two overlapping open reading frames - ORF1a and 1b. Following uncoating of the RNA genome in the cell cytoplasm, the replicase gene is translated as either an ORF 1a polyprotein (495 kD) or as an ORF1ab fusion polyprotein (803 kD), with translation of ORF 1b requiring a ribosomal frameshift event at the end of ORF 1a. The intact replicase polyproteins are not detected during natural infection, since maturation proteolytic cleavages occur cotranslationally by three proteinase functions encoded in ORF 1a polyprotein. The proteolytic processing results in 15 mature proteins, including the proteinases, an RNA helicase, and a putative RNA-dependent RNA polymerase. The MHV proteins are set forth in Table 1, and a schematic comparing MHV replicase polyprotein with a SARS strain replicase polyprotein is given in FIG. 8.

TABLE 1 – MHV Replicase Polyproteins

<u>Protein Designation</u>	<u>Residues</u>	<u>Function</u>
p28	1-247	Unknown; localizes to replication complexes early in infection where it associates with membranes by easily disrupted peripheral mechanisms
p65	248-834	Unknown; shown to associate throughout infection with membranes of replication complexes at sites of viral RNA synthesis, likely by interactions with other proteins
P210	833-2837	Encodes a protein with two papain-like proteinase domains that cleave the first three (C1-C3) cleavage sites
MP1	2838-3332	Highly hydrophobic, membrane associated, found in replication complexes
3CLpro	3333-3633	Picornain like proteinase responsible for cleavage at C4-C14
MP2		Highly hydrophobic, membrane associated, localization in cells unknown
p10		Associates with p22, p12, p15 in replication complexes
p22		Associates with p10, p12, p15 in replication complexes
p12		Associates with p22, p10, p15 in replication complexes
p15		Associates with p22, p12, p10 in replication complexes
Polymerase (pol, p100)		Putative RNA dependent RNA polymerase, localizes to replication complexes
Helicase (hel, p67)		RNA unwinding and NTPase activities
p57		Unknown
p42		Unknown
p33		Unknown

The replicase gene expresses all of the viral factors required for all stages of MHV mRNA synthesis and replication. In addition, it has been shown that inhibition of polyprotein processing at any time during infection results in rapid shutoff of viral RNA synthesis, indicating that at least some of the proteolytic processing events are required for RNA synthesis. However  
 5 there are differences in mature replicase proteins among different coronaviruses, particularly in the amino-terminal 100 kD of the polyproteins.

#### B. Cleavage Sites

By convention, the present invention identifies the polyprotein cleavage sites as C1-C14.  
 10 These sites are defined as cleaving between adjacent products. Examples for MHV are set forth in Table 2, below.

**TABLE 2 – Replicase Polyprotein Cleavage Sites**

<u>Cleavage Site</u>	<u>Upstream Protein</u>	<u>Downstream Protein</u>
C1	p28	p65
C2	p65	p210
C3	P210	MP1
C4	MP1	3C
C5	3C	MP2
C6	MP2	p10
C7	p10	p22
C8	p22	p12
C9	p12	p15
C10	p15	Polymerase
C11	Polymerase	Helicase
C12	Helicase	p57
C13	p57	p42
C14	p42	p33

15 The third protein processed from the replicase polyprotein is p210 (Schiller *et al.*, 1998). The p210 protein incorporates amino acids 833 to a predicted carboxy-terminus at amino acid

2837, with a predicted mass of 221 kD. p210 contains the two papain-like proteinase domains (PLP1 and PLP2) that have been shown to cleave the first three cleavage sites (CS1, CS2 and CS3) at the carboxy-termini of p28, p65 and p210, respectively. The apparent difference between coronaviruses in the predicted number of proteinases, and the differences in the size and number of proteins in the amino-terminal half of the polyprotein, was interpreted to indicate a lack of common critical functions in this region of gene. A recent study used sequence comparisons, parsimony analyses, and studies of the cleavage sites and proteinase functions to compare the coronavirus p210 and the corresponding p195 proteins of the human coronavirus 229E (HCoV-229E) and infectious bronchitis virus (IBV) (Ziebuhr *et al.*, 2001). The analyses identified common domains of the coronavirus p210/p195 proteins (FIG. 2), several of which had previously been predicted or confirmed for MHV (Lee *et al.*, 1991). The amino-terminal domain of p210 was referred to as the "acidic domain" (Ac) based on the concentration of acidic residues. The PLP1 domain consists of the sequence required for proteinase activity during *in vitro* cleavage reactions (Bonilla *et al.* 1995). The X domain is a region of increased conservation among the different coronavirus p210/p195 proteins with no known or predicted functions (Lee *et al.*, 1991). The functional PLP2 domains are a variable distance from the X domains, and have been less completely characterized as to their functional requirements. Both PLP1 and PLP2 have been demonstrated to function with a catalytic dyad of Cys and His residues (Baker *et al.*, 1993; Bonilla *et al.*, 1995; Kanjanahaluethai and Baker, 2000). Finally, a Y domain consists of a region incorporating two stretches of predominantly hydrophobic residues that predict membrane-spanning helices (Lee *et al.*, 1991).

Coronavirus PLPs have a zinc finger motif in the predicted papain-like fold of the enzymes, with predicted similarities to the human transcription elongation factor TFIIS (Herold *et al.*, 1999). The zinc finger has been shown to bind zinc, which is required for PLP function *in vitro*. Mutations in this motif abolish proteolytic activity. It has been suggested based on these features and demonstrated contributions of the zinc finger to RNA synthesis in the arterivirus, equine arteritis virus (EAV) (Tijms *et al.*, 2001), that the zinc finger may serve functions in addition to PLP proteolytic activity.

Studies of PLP1 and PLP2, as well as identification and detailed mutagenesis of replicase polyprotein cleavage sites, have been performed *in vitro*. PLP1 has been shown to proteolytically process the first two cleavage sites in the MHV replicase polyprotein: between

p28 and p65 at 247G/V248 (referred to as CS1) and between p65 and p210 at 832A/G833 (CS2) (Dong and Baker, 1994; Hughes *et al.*, 1995; Bonilla *et al.*, 1997; Baker *et al.*, 1993). PLP2 has been shown to cleave at the carboxy-terminus of p210 (CS3), likely in a cis autocatalytic cleavage (Kanjanaaluethai and Baker, 2000; Kanjanaaluethai and Baker, 2001). Although the MHV CS3 cleavage site has not been reported, by direct comparison with identified IBV PLP2 cleavage site the MHV-A59 p210 carboxy-terminal cleavage (CS3) would be predicted to be 2837G/A2838. Analysis of the MHV CS1 and CS2 in comparison with other group 1 coronaviruses (TGEV, HCoV-229E) (Elcouet *et al.*, 1995; Herold *et al.*, 1993), group 2 coronaviruses (MHV-JHM, BCV) (Yoo and Pei, 2001; Chouljenko *et al.*, 2001), and group 3 coronaviruses (IBV) (Bournsnel *et al.*, 1987), has demonstrated similarities at the P1/P1' cleavage dipeptides; Gly or Ala at P1 of all coronavirus PLP CS, and Val, Ala or Gly at P1'. HCoV is the exception, using Asn in the P1' position. Overall, P5, P2, P1 and P1' have been most intolerant of changes, with mutations at these sites disrupting cleavage *in vitro*.

Analysis of the coronavirus PLPs and their cognate cleavage sites suggests that PLP1 and PLP2 are paralogous proteinases, originating from a common coronavirus or pre-coronavirus ancestor, and that they have diverged over time (Ziebuhr *et al.*, 2001) (FIG. 3). For example, all coronaviruses except IBV express both PLP1 and PLP2 activities and share the common feature that PLP1 cleaves CS1 and CS2. IBV only expresses a PLP2 that cleaves at a single site equivalent to CS2. In IBV, a functional PLP1 is not detected, whereas a residual, highly altered and inactive PLP1 domain has recently been identified by sequence comparison (Ziebuhr *et al.*, 2001). These observations have led to the hypothesis that there may be overlap of cleavage site specificity and PLP activity, and possible redundancy of cleavage activity, with PLP 2 able to mediate cleavages at PLP1 cognate sites. This has been demonstrated to be true for HCoV, with both PLP1 and PLP2 able to cleave CS2 *in vitro* (Ziebuhr *et al.*, 2001). In fact, the data suggest that the "normal" CS2 cleavage event may involve the cooperative activity of PLP1 and PLP2. However, it was also demonstrated that when PLP1 was catalytically inactivated, PLP2 was able to independently mediate CS2 cleavage *in vitro*.

#### IV. Engineering of Nidovirus Genomes

Thus, in accordance with the present invention, it will be desirable to create a variety of different cleavage mutants in Nidovirus replicase polyproteins. Mutagenesis is the process whereby changes occur in the structure of a genome. Mutation can involve modification of the nucleotide sequence of a single gene, blocks of genes or a whole chromosome. Changes in single genes may be the consequence of point mutations which involve the removal, addition or substitution of a single nucleotide base within a DNA sequence, or they may be the consequence of changes involving the insertion or deletion of large numbers of nucleotides.

Mutations can arise spontaneously as a result of events such as errors in the fidelity of DNA replication or the movement of transposable genetic elements (transposons) within the genome. They also are induced following exposure to chemical or physical mutagens. Such mutation-inducing agents include ionizing radiations, ultraviolet light and a diverse array of chemical such as alkylating agents and polycyclic aromatic hydrocarbons all of which are capable of interacting either directly or indirectly (generally following some metabolic biotransformations) with nucleic acids. The DNA lesions induced by such environmental agents may lead to modifications of base sequence when the affected DNA is replicated or repaired and thus to a mutation. Mutation also can be site-directed through the use of particular targeting methods. Any number of different mutagenic approaches may be taken, as described below.

##### A. Nidovirus Genomes

One of skill in the art may use various Nidovirus replicase sequences to design specific mutations that abolish or reduce replicase protein cleavage. The following constitute non-limiting examples of Nidovirus genomic accession nos., each of which are incorporated by reference: human coronavirus 229E (NC002645), SARS TOR2 (AY274119), SARS HKU-39849 (AY278491), SARS CUHK-W1 (AY278554), bovine coronavirus (BCV) (NC003045), avian infectious bronchitis virus (IBV) (NC001451), transmissible gastroenteritis virus (TGEV), (NC002306), mouse hepatitis virus (MHV) (NC001846).

##### B. Random Mutagenesis

In one embodiment, random mutagenesis may be applied. This will, of course, require an additional step of screening for the desired mutations. Screening will typically be accomplished

by nucleic acid hybridization (Southern or Northern blotting), sequencing, or SnP analysis, methods of which are well known to those of skill in the art.

#### i) Insertional Mutagenesis

5        Insertional mutagenesis is based on the inactivation of a gene via insertion of a known DNA fragment. Because it involves the insertion of some type of DNA fragment, the mutations generated are generally loss-of-function, rather than gain-of-function mutations. However, there are several examples of insertions generating gain-of-function mutations (Oppenheimer *et al.* 1991). Insertion mutagenesis has been very successful in bacteria and *Drosophila* (Cooley *et al.* 10        1988) and recently has become a powerful tool in corn (Schmidt *et al.* 1987); *Arabidopsis*; (Marks *et al.*, 1991; Koncz *et al.* 1990); and *Antirrhinum* (Sommer *et al.* 1990).

      Transposable genetic elements are DNA sequences that can move (transpose) from one place to another in the genome of a cell. The first transposable elements to be recognized were the Activator/Dissociation elements of *Zea mays* (McClintock, 1957). Since then, they have 15        been identified in a wide range of organisms, both prokaryotic and eukaryotic.

      Transposable elements in the genome are characterized by being flanked by direct repeats of a short sequence of DNA that has been duplicated during transposition and is called a target site duplication. Virtually all transposable elements whatever their type, and mechanism of transposition, make such duplications at the site of their insertion. In some cases the number of 20        bases duplicated is constant, in other cases it may vary with each transposition event. Most transposable elements have inverted repeat sequences at their termini. These terminal inverted repeats may be anything from a few bases to a few hundred bases long and in many cases they are known to be necessary for transposition.

      Prokaryotic transposable elements have been most studied in *E. coli* and Gram negative 25        bacteria, but also are present in Gram positive bacteria. They are generally termed insertion sequences if they are less than about 2 kB long, or transposons if they are longer. Bacteriophages such as mu and D108, which replicate by transposition, make up a third type of transposable element. Elements of each type encode at least one polypeptide a transposase, required for their own transposition. Transposons often further include genes coding for function 30        unrelated to transposition, for example, antibiotic resistance genes.

Transposons can be divided into two classes according to their structure. First, compound or composite transposons have copies of an insertion sequence element at each end, usually in an inverted orientation. These transposons require transposases encoded by one of their terminal IS elements. The second class of transposon have terminal repeats of about 30  
 5 base pairs and do not contain sequences from IS elements.

Transposition usually is either conservative or replicative, although in some cases it can be both. In replicative transposition, one copy of the transposing element remains at the donor site, and another is inserted at the target site. In conservative transposition, the transposing element is excised from one site and inserted at another.

10 Eukaryotic elements also can be classified according to their structure and mechanism of transportation. The primary distinction is between elements that transpose *via* an RNA intermediate, and elements that transpose directly from DNA to DNA.

Elements that transpose *via* an RNA intermediate often are referred to as retrotransposons, and their most characteristic feature is that they encode polypeptides that are  
 15 believed to have reverse transcriptionase activity. There are two types of retrotransposon. Some resemble the integrated proviral DNA of a retrovirus in that they have long direct repeat sequences, long terminal repeats (LTRs), at each end. The similarity between these retrotransposons and proviruses extends to their coding capacity. They contain sequences related to the *gag* and *pol* genes of a retrovirus, suggesting that they transpose by a mechanism related to  
 20 a retroviral life cycle. Retrotransposons of the second type have no terminal repeats. They also code for *gag*- and *pol*-like polypeptides and transpose by reverse transcription of RNA intermediates, but do so by a mechanism that differs from that of retrovirus-like elements. Transposition by reverse transcription is a replicative process and does not require excision of an element from a donor site.

25 Transposable elements are an important source of spontaneous mutations, and have influenced the ways in which genes and genomes have evolved. They can inactivate genes by inserting within them, and can cause gross chromosomal rearrangements either directly, through the activity of their transposases, or indirectly, as a result of recombination between copies of an element scattered around the genome. Transposable elements that excise often do so imprecisely  
 30 and may produce alleles coding for altered gene products if the number of bases added or deleted is a multiple of three.

Transposable elements themselves may evolve in unusual ways. If they were inherited like other DNA sequences, then copies of an element in one species would be more like copies in closely related species than copies in more distant species. This is not always the case, suggesting that transposable elements are occasionally transmitted horizontally from one species to another.

## ii) Chemical Mutagenesis

Chemical mutagenesis offers certain advantages, such as the ability to find a full range of mutant alleles with degrees of phenotypic severity, and is facile and inexpensive to perform. The majority of chemical carcinogens produce mutations in DNA. Benzo[a]pyrene, N-acetoxy-2-acetyl aminofluorene and aflatoxin B1 cause GC to TA transversions in bacteria and mammalian cells. Benzo[a]pyrene also can produce base substitutions such as AT to TA. N-nitroso compounds produce GC to AT transitions. Alkylation of the O4 position of thymine induced by exposure to n-nitrosourea results in TA to CG transitions.

A high correlation between mutagenicity and carcinogenicity is the underlying assumption behind the Ames test (McCann *et al.*, 1975) which speedily assays for mutants in a bacterial system, together with an added rat liver homogenate, which contains the microsomal cytochrome P450, to provide the metabolic activation of the mutagens where needed.

In vertebrates, several carcinogens have been found to produce mutation in the *ras* proto-oncogene. N-nitroso-N-methyl urea induces mammary, prostate and other carcinomas in rats with the majority of the tumors showing a G to A transition at the second position in codon 12 of the Ha-ras oncogene. Benzo[a]pyrene-induced skin tumors contain A to T transformation in the second codon of the Ha-ras gene.

## iii) Radiation Mutagenesis

The integrity of biological molecules is degraded by the ionizing radiation. Adsorption of the incident energy leads to the formation of ions and free radicals, and breakage of some covalent bonds. Susceptibility to radiation damage appears quite variable between molecules, and between different crystalline forms of the same molecule. It depends on the total accumulated dose, and also on the dose rate (as once free radicals are present, the molecular

damage they cause depends on their natural diffusion rate and thus upon real time). Damage is reduced and controlled by making the sample as cold as possible.

Ionizing radiation causes DNA damage and cell killing, generally proportional to the dose rate. Ionizing radiation has been postulated to induce multiple biological effects by direct  
5 interaction with DNA, or through the formation of free radical species leading to DNA damage (Hall, 1988). These effects include gene mutations, malignant transformation, and cell killing. Although ionizing radiation has been demonstrated to induce expression of certain DNA repair genes in some prokaryotic and lower eukaryotic cells, little is known about the effects of ionizing radiation on the regulation of mammalian gene expression (Borek, 1985). Several studies have  
10 described changes in the pattern of protein synthesis observed after irradiation of mammalian cells. For example, ionizing radiation treatment of human malignant melanoma cells is associated with induction of several unidentified proteins (Boothman *et al.*, 1989). Synthesis of cyclin and co-regulated polypeptides is suppressed by ionizing radiation in rat REF52 cells, but not in oncogene-transformed REF52 cell lines (Lambert and Borek, 1988). Other studies have  
15 demonstrated that certain growth factors or cytokines may be involved in x-ray-induced DNA damage. In this regard, platelet-derived growth factor is released from endothelial cells after irradiation (Witte *et al.*, 1989).

In the present invention, the term "ionizing radiation" means radiation comprising particles or photons that have sufficient energy or can produce sufficient energy *via* nuclear  
20 interactions to produce ionization (gain or loss of electrons). An exemplary and preferred ionizing radiation is an x-radiation. The amount of ionizing radiation needed in a given cell generally depends upon the nature of that cell. Typically, an effective expression-inducing dose is less than a dose of ionizing radiation that causes cell damage or death directly. Means for determining an effective amount of radiation are well known in the art.

25 In a certain embodiments, an effective expression inducing amount is from about 2 to about 30 Gray (Gy) administered at a rate of from about 0.5 to about 2 Gy/minute. Even more preferably, an effective expression inducing amount of ionizing radiation is from about 5 to about 15 Gy. In other embodiments, doses of 2-9 Gy are used in single doses. An effective dose of ionizing radiation may be from 10 to 100 Gy, with 15 to 75 Gy being preferred, and 20 to 50  
30 Gy being more preferred.

Any suitable means for delivering radiation to a tissue may be employed in the present invention in addition to external means. For example, radiation may be delivered by first providing a radiolabeled antibody that immunoreacts with an antigen of the tumor, followed by delivering an effective amount of the radiolabeled antibody to the tumor. In addition,  
5 radioisotopes may be used to deliver ionizing radiation to a tissue or cell.

iv) *In Vitro* Scanning Mutagenesis

Random mutagenesis also may be introduced using error prone PCR (Cadwell and Joyce, 1992). The rate of mutagenesis may be increased by performing PCR in multiple tubes with  
10 dilutions of templates.

One particularly useful mutagenesis technique is alanine scanning mutagenesis in which a number of residues are substituted individually with the amino acid alanine so that the effects of losing side-chain interactions can be determined, while minimizing the risk of large-scale perturbations in protein conformation (Cunningham *et al.*, 1989).

15 In recent years, techniques for estimating the equilibrium constant for ligand binding using minuscule amounts of protein have been developed (Blackburn *et al.*, 1991; U.S. Patents 5,221,605 and 5,238,808). The ability to perform functional assays with small amounts of material can be exploited to develop highly efficient, *in vitro* methodologies for the saturation mutagenesis of antibodies. The inventors bypassed cloning steps by combining PCR mutagenesis  
20 with coupled *in vitro* transcription/translation for the high throughput generation of protein mutants. Here, the PCR products are used directly as the template for the *in vitro* transcription/translation of the mutant single chain antibodies. Because of the high efficiency with which all 19 amino acid substitutions can be generated and analyzed in this way, it is now possible to perform saturation mutagenesis on numerous residues of interest, a process that can  
25 be described as *in vitro* scanning saturation mutagenesis (Burks *et al.*, 1997).

*In vitro* scanning saturation mutagenesis provides a rapid method for obtaining a large amount of structure-function information including: (i) identification of residues that modulate ligand binding specificity, (ii) a better understanding of ligand binding based on the identification of those amino acids that retain activity and those that abolish activity at a given  
30 location, (iii) an evaluation of the overall plasticity of an active site or protein subdomain, (iv) identification of amino acid substitutions that result in increased binding.

v) Random Mutagenesis by Fragmentation and Reassembly

A method for generating libraries of displayed polypeptides is described in U.S. Patent 5,380,721. The method comprises obtaining polynucleotide library members, pooling and  
5 fragmenting the polynucleotides, and reforming fragments therefrom, performing PCR amplification, thereby homologously recombining the fragments to form a shuffled pool of recombined polynucleotides.

C. Site-Directed Mutagenesis

10 Structure-guided site-specific mutagenesis represents a powerful tool for the dissection and engineering of protein-ligand interactions (Braisted and Wells, 1996), especially in the context of the present invention where specific mutations in cleavage sites are sought. The technique provides for the preparation of sequence variants by introducing one or more discrete nucleotide sequence changes into a selected nucleic acid.

15 Site-specific mutagenesis uses specific oligonucleotide sequences which encode the DNA sequence of the desired mutation, as well as a sufficient number of adjacent, unmodified nucleotides. In this way, a primer sequence is provided with sufficient size and complexity to form a stable duplex on both sides of the deletion junction being traversed. A primer of about 17 to 25 nucleotides in length is preferred, with about 5 to 10 residues on both sides of the junction  
20 of the sequence being altered.

The technique typically employs a bacteriophage vector that exists in both a single-stranded and double-stranded form. Vectors useful in site-directed mutagenesis include vectors such as the M13 phage. These phage vectors are commercially available and their use is generally well known to those skilled in the art. Double-stranded plasmids are also routinely  
25 employed in site-directed mutagenesis, which eliminates the step of transferring the gene of interest from a phage to a plasmid.

In general, one first obtains a single-stranded vector, or melts two strands of a double-stranded vector, which includes within its sequence a DNA sequence encoding the desired protein or genetic element. An oligonucleotide primer bearing the desired mutated sequence,  
30 synthetically prepared, is then annealed with the single-stranded DNA preparation, taking into account the degree of mismatch when selecting hybridization conditions. The hybridized

product is subjected to DNA polymerizing enzymes such as *E. coli* polymerase I (Klenow fragment) in order to complete the synthesis of the mutation-bearing strand. Thus, a heteroduplex is formed, wherein one strand encodes the original non-mutated sequence, and the second strand bears the desired mutation. This heteroduplex vector is then used to transform appropriate host cells, such as *E. coli* cells, and clones are selected that include recombinant vectors bearing the mutated sequence arrangement.

Comprehensive information on the functional significance and information content of a given residue of protein can best be obtained by saturation mutagenesis in which all 19 amino acid substitutions are examined. The shortcoming of this approach is that the logistics of multiresidue saturation mutagenesis are daunting (Warren *et al.*, 1996; Zeng *et al.*, 1996; Barbas *et al.*, 1994; Yelton *et al.*, 1995; Wong *et al.*, 1996; Hilton *et al.*, 1996). Hundreds, and possibly even thousands, of site specific mutants must be studied. However, improved techniques make production and rapid screening of mutants much more straightforward. See also, U.S. Patents 5,798,208 and 5,830,650, for a description of "walk-through" mutagenesis.

Other methods of site-directed mutagenesis are disclosed in U.S. Patents 5,220,007; 5,284,760; 5,354,670; 5,366,878; 5,389,514; 5,635,377; and 5,789,166.

#### **D. Virus Transformation and Propagation**

Targeted recombination has become a powerful tool to introduce mutations into the genome and determine their effects on protein function, virus replication and virus pathogenesis (Koetzner *et al.*, 1992; Masters *et al.*, 1994; Fischer *et al.*, 1997; Lavi *et al.*, 1998; Leparc-Goffart *et al.*, 1998; Phillips *et al.*, 1999; Sanchez *et al.*, 1999; Phillips *et al.*, 2001; de haan *et al.*, 2002; Sarma *et al.*, 2002). However, the available recombination constructs and methodologies have thus far limited the use of targeted recombination, and have not been employed to examine mutations in the replicase gene.

The inventors have previously collaborated in the development of a system for assembly of full-length MHV genome cDNA, generation of genome length RNA, and recovery of virus from transfected cells (Schaad *et al.*, 1990; Yount *et al.*, 2002). In this process, seven contiguous cDNA clones that spanned the 31.5-kb genome of mouse hepatitis virus strain A59 (MHV-A59) were isolated. The ends of the cDNAs were engineered with unique junctions and assembled with only the adjacent cDNA subclones, resulting in an intact MHV-A59 cDNA construct of

about 31.5 kb in length. The interconnecting restriction site junctions that are located at the ends of each cDNA are systematically removed during the assembly of the complete full-length cDNA product, allowing reassembly without the introduction of nucleotide changes.

RNA transcripts derived from the full-length MHV-A59 construct were infectious, although transfection frequencies were enhanced 10- to 15-fold in the presence of transcripts encoding the nucleocapsid protein N. Plaque-purified virus derived from the infectious construct replicated efficiently and displayed similar growth kinetics, plaque morphology, and cytopathology in murine cells as did wild-type MHV-A59. Molecularly cloned viruses recognized the MHV receptor (MHVR) for docking and entry, and pretreatment of cells with monoclonal antibodies against MHVR blocked virus entry and replication. Cells infected with molecularly cloned MHV-A59 virus expressed replicase (gene 1) proteins identical to those of laboratory MHV-A59. Importantly, the molecularly cloned viruses contained three marker mutations that had been derived from the engineered component clones.

Using this process, full-length infectious constructs of MHV-A59 and other coronaviruses with genetic modifications of may be created. In fact, the method has the potential to be used to construct viral, microbial, or eukaryotic genomes approaching several million base pairs in length and used to insert restriction sites at any given nucleotide in a microbial genome. A similar system approach was used previously with TGEV, including the insertion of heterologous genes into the TGEV genome (Yount, 2000; Curtis *et al.*, 2002). The inventors described herein the use of this same assembly approach to introduce five different mutations into the MHV p28/p65 cleavage site (CS1). While the approaches are similar, it was not usually necessary with MHV to introduce mutations and new restriction sites into the wild-type virus genome to direct the assembly cascade. Rather, type IIS restriction endonuclease *Esp3I* sites can be used to create the unique interconnecting junctions, and yet be subsequently removed from the final assembly product, allowing for the reconstruction of an intact wild-type sequence. This approach avoids the introduction of nucleotide changes that are normally associated with building a full-length cDNA product of a viral genome.

The use of non-palindromic restriction sites also provides other novel recombinant DNA applications. For example, by PCR, it is possible to insert *Esp3I* or a related non-palindromic restriction site at any given nucleotide in a viral genome and use the variable domain for simple and rapid site-specific mutagenesis. By orienting the restriction sites as No See'm, the sites are

removed during reassembly, leaving only the desired mutation in the final DNA product. The dual properties of strand specificity and a variable end overhang that can be tailored to match any sequence allow for *Esp3I* sites to be engineered as universal connectors that can be joined with any other 4-nucleotide restriction site overhang (e.g., *EcoRI*, *PstI*, and *BamHI*). Alternatively, No See'm sites can be used to insert foreign genes into viral, eukaryotic, or microbial genomes or vectors, simultaneously removing all evidence of the restriction sites that were used in the recombinant DNA manipulation.

In order to remove preexisting *Esp3I* sites that resided within the MHV-A59 genome sequence, silent mutations were created. This helped to distinguish between molecularly cloned and wild-type viruses. In one instance, the *Esp3I* site at position 4875 was removed because it left a TTAA overhang that would have prevented the directionality of assembly. The other *Esp3I* sites were removed to minimize the total number of MHV-A59 subclones used in the assembly cascade. In two instances, silent mutations were inserted into the *Esp3I* overhang to maximize sequence specificity and directionality at a particular junction, but this could be circumvented by choosing slightly different junction sites. Clearly, each virus sequence will need to be evaluated for the need for similar changes.

cDNA cassettes can be ligated systematically as previously described for TGEV, or simultaneously as described herein. Although numerous incomplete assembly intermediates occur were evident, the inventors have found that simultaneous ligation of seven cDNAs will result in full-length cDNA, thereby simplifying the complexity of the assembly strategy. There is no evidence to indicate that this approach might introduce spurious mutations or genome rearrangements from aberrant assembly cascades. And while it is possible that such variants might arise following RNA transfection (as a consequence of high-frequency MHV RNA recombination between incomplete and genome-length transcripts), it is highly likely that such variants would be replication impaired and rapidly outcompeted by wild-type virus. A second limitation is that the yield of full-length cDNA product is reduced, resulting in less robust transfection efficiencies than those of the more traditional systematic assembly method. This downside is more than compensated by the reduced complexity in many cases.

## V. Vaccines

### A. Formulations and Administration

The present invention provides for Nidovirus vaccine formulations. Such compositions will generally be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium. There are numerous examples of vaccine formulations in the literature, and one of skill  
5 in the art will be capable of formulating such vaccines.

The phrases "pharmaceutically acceptable" or "pharmacologically acceptable" refer to molecular entities and compositions that do not produce an adverse, allergic, or other untoward reaction when administered to an animal, or human, as appropriate. As used herein,  
10 "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutical active substances is well known in the art.

The vaccines of the present invention can be formulated for parenteral administration, *e.g.*, formulated for injection *via* the intravenous, intramuscular, sub-cutaneous, or even  
15 intraperitoneal routes. Typically, such compositions can be prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for use to prepare solutions or suspensions upon the addition of a liquid prior to injection can also be prepared; and, the preparations can also be emulsified. The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil, or aqueous propylene  
20 glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases the form must be sterile and must be fluid to the extent that it may be easily injected. It also should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

The carrier also can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion, and by the use of surfactants. The prevention of the action of  
25 microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it  
30

will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminum monostearate and gelatin.

Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques. In certain cases, the therapeutic formulations of the invention also may be prepared in forms suitable for oral or intranasal administration.

An effective amount of the vaccine is determined based on the intended goal. The term "unit dose" or "dosage" refers to physically discrete units suitable for use in a subject, each unit containing a predetermined-quantity of the composition calculated to produce the desired responses, discussed above, in association with its administration, *i.e.*, the appropriate route and treatment regimen. Precise amounts of the vaccine composition also depend on the judgment of the practitioner and are peculiar to each individual. Factors affecting dose include physical and clinical state of the patient, the route of administration, the intended goal of treatment (alleviation of symptoms *versus* cure) and the potency, stability, and toxicity of the particular substance.

The following is a listing of references describing various live vaccines, the relevant contents of which (formulations and administration) are hereby incorporated by reference:

U.S. Patents 6,479,056 ; 6,444,445; 6,306,400; 6,296,854; 6,231,871; 6,217,882; 6,159,477; 6,153,199; 6,136,325; 6,077,516; 6,051,237; 6,045,803; 6,039,958; 6,039,941; 6,033,670; 5,993,822 ; 5,980,906; 5,958,423; 5,948,411; 5,871,742; 5,869,036; 5,792,452; 5,733,555; 5,733,554; 5,651,972; 5,632,989; 5,626,850; 5,580,557; 5,436,001; 5,310,668; 5,149,531; 5,068,104; 5,037,650; 5,024,836; 5,006,335; 4,985,244; 4,980,162; 4,808,404; 4,770,875; 4,762,711; 4,752,474; 4,673,572; 4,645,665; 4,624,850; 4,590,072; 4,555,401; 4,554,158; 4,472,378; 4,456,588; 4,324,861; 4,311,797; 4,235,876; 4,004,974

#### **B. Additional Agents**

In addition to the inactive agents discussed above, the vaccine may comprise, or may be given in conjunction with, a supplemental agent. One example is an immunostimulant.

## VI. Examples

The following examples are included to further illustrate various aspects of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples which follow represent techniques and/or compositions discovered by the inventor to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments which are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention.

### EXAMPLE 1: MATERIALS & METHODS

The inventors used a recently developed reverse genetic system to introduce mutations into the cleavage site between the first two protein domains of the replicase polypeptide, p28 and p65. The mutations were based on *in vitro* studies that have carefully defined mutations in the p28-p65 cleavage site that either retain or abolish p28-p65 cleavage. The mutated genome fragments were used to assemble a full-length genomic cDNA, which was then transcribed into positive-sense genome RNA and electroporated into BHK cells expressing the MHV receptor.

Virus cytopathic effect (CPE) was detected for assembled wild-type MHV (icMHV), mutants with predicted p28-p65 cleavage (icMut9), and three mutants with predicted cleavage knockout mutations (icMut 3,4,5).

The inventors obtained virus stocks from plaque-cloned viruses, and used them in biochemical, virus growth and cell imaging experiments. When the genomic RNA was RT-PCR sequenced across the first 5 kB, all of the wild-type and mutant viruses were found to have the correct nucleotide sequence.

### EXAMPLE 2: RESULTS

To define the protein expression and processing of viral mutants, infected cells were radiolabeled, and cell lysates were immunoprecipitated using antibodies to detect p28 and p65 (47) (FIGS. 6A-B). The laboratory MHV (L-MHV), icMHV and predicted cleavage-competent icMut8 and icMut9 mutants all had identical patterns, with distinct p28 and p65 protein bands, and no evidence of an uncleaved p28-p65 precursor. In cells infected with predicted cleavage

knockout mut3, 4, and 5 viruses, no p28 or p65 was detected; instead a new band of 93 kD was identified, consistent with an uncleaved p28-p65 precursor (FIG. 6A). A faint band was detected in infected cells with the mobility of p28, but this was shown also to be detectable in mock-infected cells following IP with anti-p28 antibodies and in infected cells immunoprecipitated with preimmune serum (FIG. 6B). No p28 or p65 was detected even following prolonged chase, indicating that cleavage was blocked rather than decreased in rate. The patterns in both sets of mutants were consistent with the results of in vitro translation and processing, and together with the sequence analysis, demonstrated that the mutations were present and resulted in the inhibition of protein processing for the knockout viruses ic Mut 3, 4, and 5.

Next, virus growth was determined in both single-cycle (high multiplicity of infection ("MOI")) and multiple cycle (low MOI) growth experiments in murine DBT cells. In single-cycle growth experiments, the icMut 8, and 9 cleavage competent mutants had growth kinetics and peak titers identical to L-MHV and assembled icMHV. In contrast, icMut 3, 4, and 5 had both delayed growth and diminished peak titers at 12 h, an effect that was amplified at 24 h. During plaque assays, the plaque size for the cleavage knockout mutants was consistently visibly smaller than those of L-MHV, icMHV and icMut 8, and 9. Images of plaques were obtained and measured, with plaques from cleavage knockout mutants averaging a 75% reduction in size at 30 h p.i. compared to icMHV and cleavage competent mutants. Both the growth kinetics and plaque morphology were suggestive of substantially impaired replication. However, if the cell monolayers were observed for longer periods, they became completely involved with virus CPE. Growth curves for the cleavage knockout mutants were similar in shape to wild-type and cleavage competent mutants but were delayed in time and lessened in peak titer, suggesting that the replication defect introduced by inhibition of p28-p65 cleavage is a qualitative one, but that all stages of virus replication can be mediated during infection with the virus mutants.

To determine if the replication defect was associated with a change in the intracellular localization of uncleaved p93, as compared with cleaved p28 and p65, infected cells on glass coverslips were probed using antibodies against p28, p65, and N. In wild-type MHV-infected cells, p28 colocalized with p65 and the structural N (nucleocapsid protein) in widely distributed cytoplasmic viral replication complexes (FIGS. 7A-C). When cells infected with the cleavage knockout mutant virus icMut 5 were probed, the specific antibodies against p65 detected the p93 protein colocalizing with N in replication complexes. In contrast to p28 and p65, p93 was also

detected in the cytoplasm distinct from replication complexes, suggesting that protein associations of the uncleaved p93 precursor may be altered compared to p28 and p65. This result further corroborated the qualitative nature of the replication defect of the cleavage knockout mutants in growth, and plaque size.

5        The mutations at the p28-p65 cleavage site were 1 to 4 nucleotide changes, and it was predicted that they would be subject to rapid reversion under selective pressure for enhanced growth in culture. Cleavage site mutants were passaged 10 times at 12-18 hr intervals, and the sequences genome RNA from of initial input and passage-10 viruses were compared. All mutants retained the original mutations, and no additional mutations were noted in the 600 nt  
10 flanking the cleavage site. No change was detected in the timing and extent of viral cytopathic effect (syncytia) or plaque morphology during the passaging of virus mutants. These results indicate that the mutants are not subject to rapid compensating mutations during replication.

      The ability of MHV to replicate with complete inhibition of the first proteolytic cleavage event was surprising since coronavirus polyprotein cleavages are a strongly conserved feature  
15 across the viruses. They also are quite specific and temporally regulated during virus growth in cells, suggesting critical regulatory functions in virus replication. Clearly, virus growth was attenuated, indicating that cleavage of p28 from p65 is important for virus growth; however the replication defect in cleavage knockout mutants was not lethal. In fact, it was apparently not even deleterious enough to exert selective pressure for reversion of single point mutations in the  
20 cleavage site.

      One explanation is that p28 and p65 are able to mediate functions in an uncleaved form. Comparisons of different coronavirus groups have shown that group 2 coronaviruses (MHV, BCV, HCoV OC43) have p28 and p65 proteins with conserved cleavage sites, while the group 1 and 3 coronaviruses have a single large amino-terminal protein of 87 to 105 kD and lack a  
25 corresponding cleavage site. The analyses, and the results described herein, suggest that the p28-p65 cleavage may have arisen in a common group 2 ancestor and was retained due to a relative replication advantage in the initial animal host.

\* \* \* \* \*

30        All of the compositions and methods disclosed and claimed herein can be made and executed without undue experimentation in light of the present disclosure. While the

compositions and methods of this invention have been described in terms of preferred embodiments, it will be apparent to those of skill in the art that variations may be applied to the compositions and methods, and in the steps or in the sequence of steps of the methods described herein without departing from the concept, spirit and scope of the invention. More specifically,  
5 it will be apparent that certain agents which are both chemically and physiologically related may be substituted for the agents described herein while the same or similar results would be achieved. All such similar substitutes and modifications apparent to those skilled in the art are deemed to be within the scope of the invention as defined by the appended claims.

## VI. References

The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference:

U.S. Patent 4,004,974  
U.S. Patent 4,235,876  
U.S. Patent 4,311,797  
U.S. Patent 4,324,861  
U.S. Patent 4,456,588  
U.S. Patent 4,472,378  
U.S. Patent 4,554,158  
U.S. Patent 4,555,401  
U.S. Patent 4,590,072  
U.S. Patent 4,624,850  
U.S. Patent 4,645,665  
U.S. Patent 4,673,572  
U.S. Patent 4,752,474  
U.S. Patent 4,762,711  
U.S. Patent 4,770,875  
U.S. Patent 4,808,404  
U.S. Patent 4,980,162  
U.S. Patent 4,985,244  
U.S. Patent 5,006,335  
U.S. Patent 5,024,836  
U.S. Patent 5,037,650  
U.S. Patent 5,068,104  
U.S. Patent 5,149,531  
U.S. Patent 5,220,007  
U.S. Patent 5,221,605  
U.S. Patent 5,238,808  
U.S. Patent 5,284,760

U.S. Patent 5,310,668  
U.S. Patent 5,354,670  
U.S. Patent 5,366,878  
U.S. Patent 5,380,721  
U.S. Patent 5,389,514  
U.S. Patent 5,436,001  
U.S. Patent 5,580,557  
U.S. Patent 5,626,850  
U.S. Patent 5,632,989  
U.S. Patent 5,635,377  
U.S. Patent 5,651,972  
U.S. Patent 5,733,554  
U.S. Patent 5,733,555  
U.S. Patent 5,789,166  
U.S. Patent 5,792,452  
U.S. Patent 5,798,208  
U.S. Patent 5,830,650  
U.S. Patent 5,869,036  
U.S. Patent 5,871,742  
U.S. Patent 5,948,411  
U.S. Patent 5,958,423  
U.S. Patent 5,980,906  
U.S. Patent 5,993,822  
U.S. Patent 6,033,670  
U.S. Patent 6,039,941  
U.S. Patent 6,039,958  
U.S. Patent 6,045,803  
U.S. Patent 6,051,237  
U.S. Patent 6,077,516  
U.S. Patent 6,136,325  
U.S. Patent 6,153,199

U.S. Patent 6,159,477  
U.S. Patent 6,217,882  
U.S. Patent 6,231,871  
U.S. Patent 6,296,854  
U.S. Patent 6,306,400  
U.S. Patent 6,444,445  
U.S. Patent 6,479,056

Baker *et al.*, *J. Virol.*, 67:6056-6063, 1993.  
Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, 91(9):3809-3813, 1994.  
Blackburn *et al.*, *J. Lipid. Res.*, 32(12):1911-1918, 1991.  
Bonilla *et al.*, *J. Virol.*, 71:900-909, 1997.  
Bonilla *et al.*, *Virology*, 209:489-497, 1995.  
Boothman *et al.*, *Cancer Res.*, 49(11):2871-2878, 1989.  
Borek, *Carcinog. Compr. Surv.*, 10:303-316, 1985.  
Boursnell *et al.*, *J. Gen. Virol.*, 68:57-77, 1987.  
Braisted and Wells, *Proc. Natl. Acad. Sci. USA*, 93(12):5688-5692, 1996.  
Burks *et al.*, *Proc. Natl. Acad. Sci. USA*, 94(2):412-417, 1997.  
Caldwell and Joyce, *PCR Methods Appl.*, 2(1):28-33, 1992.  
Chouljenko *et al.*, *J. Gen. Virol.*, 82:2927-2933, 2001.  
Cooley *et al.*, *Science*, 239(4844):1121-1128, 1988.  
Cunningham and Wells, *Science*, 244(4908):1081-1085, 1989.  
Curtis *et al.*, *J. Virol.*, 76:1422-1434, 2002.  
de Haan *et al.*, *Virology*, 296:177-189, 2002.  
Dong and Baker, *Virology*, 204:541-549, 1994.  
Eleouet *et al.*, *Virology*, 206:817-822, 1995.  
Fischer *et al.*, *J. Virol.*, 71:5148-5146, 1997.  
Hall, *Genetics*, 120(4):887-897, 1988.  
Herold *et al.*, *J. Biol. Chem.*, 274:14918-14925, 1999.  
Herold *et al.*, *Virology*, 195:680-691, 1993.  
Hilton *et al.*, *J. Biol. Chem.*, 271(9):4699-4708, 1996.

- Hughes *et al.*, *J. Virol.*, 69:809-813, 1995.
- Kanjanahaluethai and Baker, *J. Virol.*, 74:7911-7921, 2000.
- Kanjanahaluethai *et al.*, *Adv. Exp. Med. Biol.*, 494:267-273, 2001.
- Koetzner *et al.*, *J. Virol.*, 66:1841-1848, 1992.
- Koncz *et al.*, *EMBO J.*, 9(5):1337-1346, 1990.
- Lambert and Borek, *J. Natl. Cancer Inst.*, 80(18):1492-1497, 1988.
- Lavi *et al.*, *Adv. Exp. Med. Biol.*, 440:543-547, 1998.
- Lee *et al.*, *Virology*, 180:567-582, 1991.
- Leparc-Goffart *et al.*, *J. Virol.*, 72:9628-9636, 1998.
- Marks *et al.*, *Symp. Soc. Exp. Biol.*, 45:77-87, 1991.
- Masters *et al.*, *J. Virol.*, 68:328-337, 1994.
- McCann *et al.*, *Proc. Natl. Acad. Sci. USA*, 72(3):979-983, 1975.
- Oppenheimer *et al.*, *Cell*, 67(3):483-493, 1991.
- Phillips *et al.*, *J. Neurovirol.*, 7:421-431, 2001.
- Phillips *et al.*, *J. Virol.*, 73:7752-7760, 1999.
- Sanchez *et al.*, *J. Virol.*, 73:7607-7618, 1999.
- Sarma *et al.*, *J. Neurovirol.*, 8:381-391, 2002.
- Schaad *et al.*, *Virology*, 177:634-645, 1990.
- Schiller *et al.*, *Virology*, 242:288-302, 1998.
- Schmidt *et al.*, *Science*, 238(4829):960-963, 1987.
- Sommer *et al.*, *EMBO J.*, 9(3):605-613, 1990.
- Tijms *et al.*, *Proc. Natl. Acad. Sci. USA*, 98:1889-1894, 2001.
- Warren *et al.*, *Biochemistry*, 35(27):8855-8862, 1996.
- Wells *et al.*, *J. Leukoc. Biol.*, 59(1):53-60, 1996.
- Witte *et al.*, *Cancer Res.*, 49(18):5066-5072, 1989.
- Wong *et al.*, *J. Bacteriol.*, 178(8):2334-2342, 1996.
- Yelton *et al.*, *J. Immunol.*, 155(4):1994-2004, 1995.
- Yoo and Pei, *Adv. Exp. Med. Biol.*, 494:73-76, 2001.
- Yount *et al.*, *J. Virol.*, 76:11065-11078, 2002.
- Yount, *J. Virol.*, 74:10600-10611, 2000.
- Zeng *et al.*, *Biochemistry*, 35(40):13157-13164, 1996.

60464456 , 042203

Ziebuhr *et al.*, *J. Virol.*, 73(1):177-185, 1999.

Ziebuhr *et al.*, *J. Biol. Chem.*, 276:33220-33232, 2001.

**WHAT IS CLAIMED IS:**

1. A live, attenuated virus of the order *Nidovirales*, said virus characterized as comprising a genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage.
2. The virus of claim 1, wherein the virus is of the family *Coronaviridae*.
3. The virus of claim 2, wherein the virus is a coronavirus or a torovirus.
4. The virus of claim 3, wherein the coronavirus is avian infectious bronchitis virus, bovine coronavirus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E, human coronavirus OC43, murine hepatitis virus, porcine epidemic diarrhea virus, porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, rat coronavirus, turkey coronavirus, severe acute respiratory syndrome virus, or rabbit coronavirus.
5. The virus of claim 3, wherein the torovirus is Berne virus or Breda virus.
6. The virus of claim 1, wherein the virus is of the family *Ateriviridae*.
7. The virus of claim 6, wherein the virus is an aterivirus.
8. The virus of claim 7, wherein the aterivirus is equine arteritis virus, Lelystad virus or simian hemorrhagic fever virus.
9. The virus of claim 1, wherein said replicase polyprotein comprises at least a second proteinase cleavage site that exhibits reduced as compared to wild-type, or no cleavage.
10. The virus of claim 4, wherein the cleavage site is a C1-C14 cleavage site.
11. The virus of claim 4, wherein the cleavage site is a murine hepatitis virus p28-p65 or p65-p210 cleavage site.
12. The virus of claim 1, wherein the cleavage site exhibits reduced cleavage as compared to wild-type.

13. The virus of claim 1, wherein the cleavage site exhibits no cleavage.
14. The virus of claim 1, wherein the cleavage site contains an amino acid deletion, an amino acid insertion or an amino acid substitution.
15. The virus of claim 1, wherein the cleavage site is wild-type, but cleavage is reduced or eliminated by an allosteric mutation.
16. A method of inducing an anti-viral immune response in a host comprising administering to said host a live, attenuated vaccine of the order *Nidovirales*, said vaccine characterized as comprising a genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage.
17. The method of claim 16, wherein the vaccine is of the family *Coronaviridae*.
18. The method of claim 17, wherein the vaccine is a coronavirus or a torovirus.
19. The method of claim 18, wherein the coronavirus is avian infectious bronchitis virus, bovine coronavirus, canine coronavirus, feline infectious peritonitis virus, human coronavirus 229E, human coronavirus OC43, murine hepatitis virus, porcine epidemic diarrhea virus, porcine hemagglutinating encephalomyelitis virus, porcine transmissible gastroenteritis virus, rat coronavirus, turkey coronavirus, severe acute respiratory syndrome virus, or rabbit coronavirus.
20. The method of claim 18, wherein the torovirus is Berne virus or Breda virus.
21. The method of claim 16, wherein the vaccine is of the family *Ateriviridae*.
22. The method of claim 21, wherein the vaccine is an aterivirus.
23. The method of claim 22, wherein the aterivirus is equine arteritis virus, Lelystad virus or simian hemorrhagic fever virus.
24. The method of claim 16, wherein said replicase polyprotein comprises at least a second proteinase cleavage site that exhibits reduced as compared to wild-type, or no cleavage.

25. The method of claim 19, wherein the cleavage site is a C1-C14 cleavage site.
26. The method of claim 19, wherein the cleavage site is a murine hepatitis virus p28-p65 or p65-p210 cleavage site.
27. The method of claim 16, wherein the cleavage site exhibits reduced cleavage as compared to wild-type.
28. The method of claim 16, wherein the cleavage site exhibits no cleavage.
29. The method of claim 16, wherein the cleavage site contains an amino acid deletion, an amino acid insertion or an amino acid substitution.
30. The method of claim 16, wherein the cleavage site is wild-type, but cleavage is reduced or eliminated by an allosteric mutation.
31. The method of claim 16, wherein said vaccine is administered intravenously or subcutaneously.
32. The method of claim 16, further comprising immunostimulant.
33. The method of claim 16, wherein said host is a dog, a cow, a pig, a cat, a mouse, a rat, a horse, a chicken, a turkey, a monkey or a human.
34. A nidovirus genome, said genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage.
35. A nidovirus replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage.
36. A vaccine comprising (a) a live, attenuated virus of the order *Nidovirales*, said virus characterized as comprising a genome encoding a replicase polyprotein comprising at least one proteinase cleavage site that exhibits reduced or no cleavage, and (b) a pharmaceutically acceptable diluent.

60464456 042203

37. The vaccine of claim 35, wherein said vaccine is formulated as a unit dose of  $10^6$  to  $10^{14}$  infectious particles.
38. The vaccine of claim 35, wherein said unit dose is provided in a 100 ml aliquot.
39. The vaccine of claim 35, further comprising a preservative.
40. The vaccine of claim 35, wherein said vaccine is lyophilized.

**ABSTRACT**

The present invention is directed live, attenuated Nidovirus vaccines, and in a particular embodiment, to coronavirus vaccines. The vaccine comprises a viral genome encoding a replicase polypprotein having at least one proteinasc cleavage site that exhibits reduced or no  
5 cleavage. Such viruses show reduced

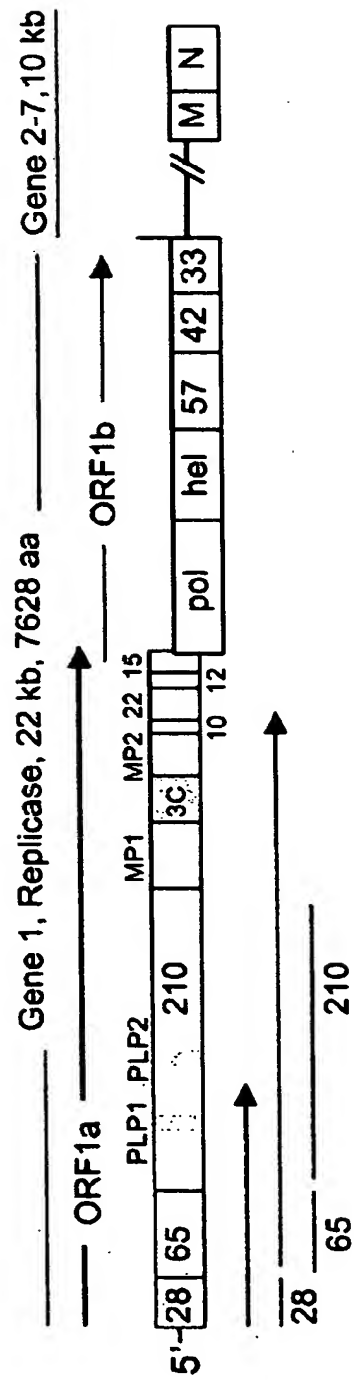


FIG. 1

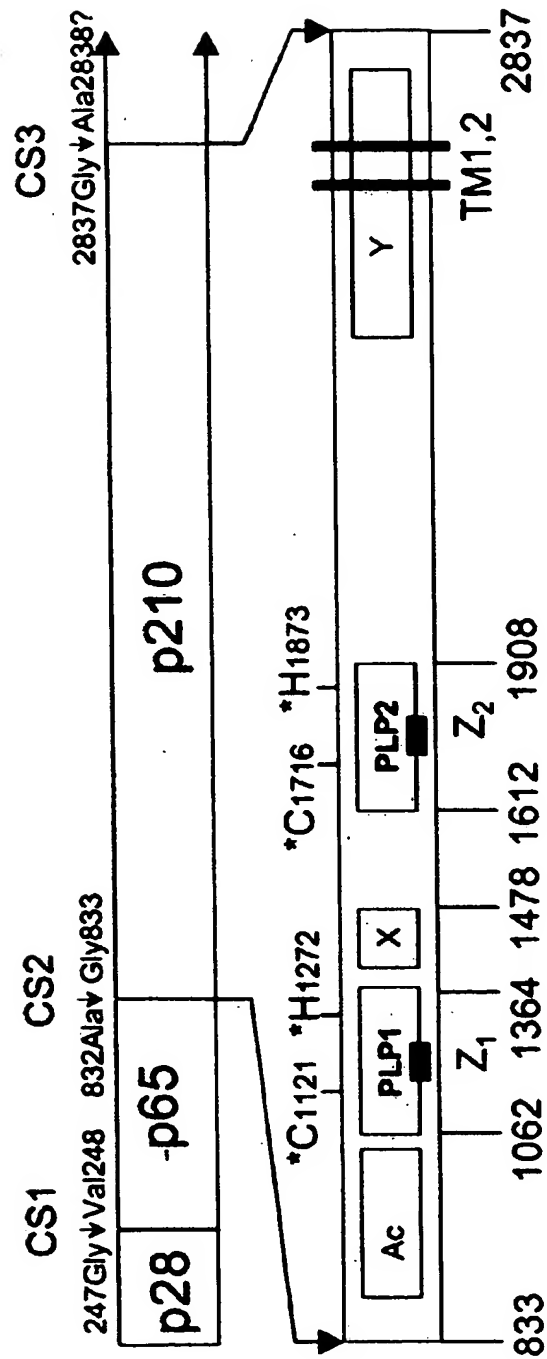


FIG. 2

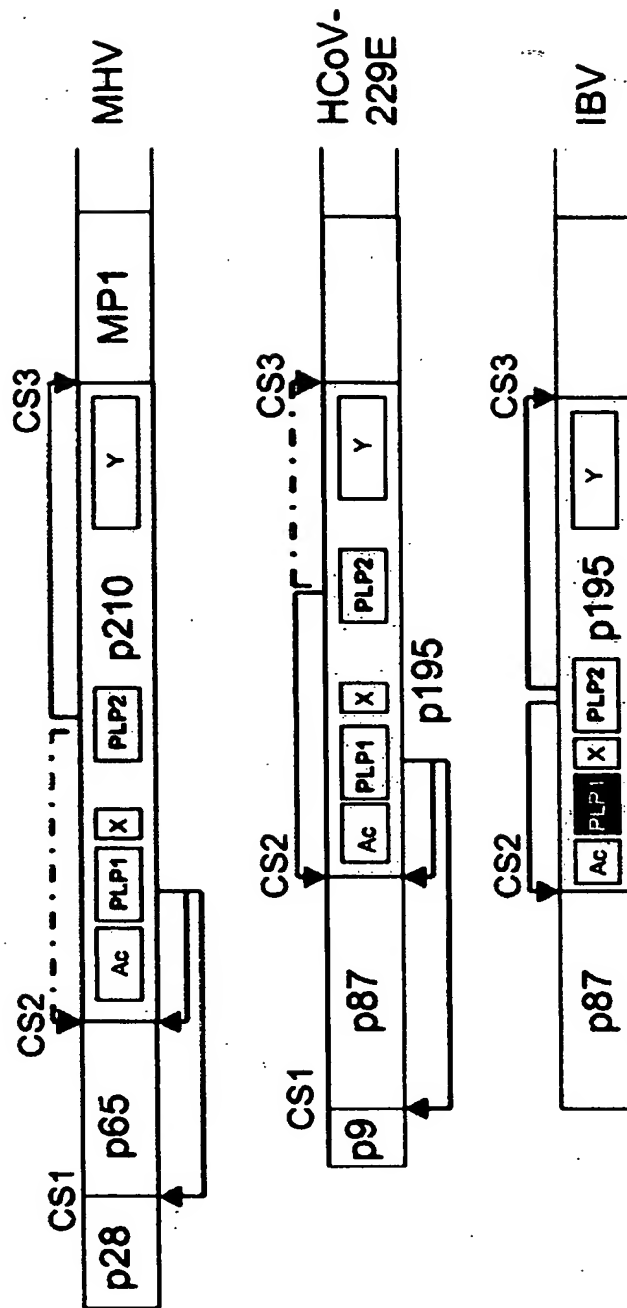


FIG. 3

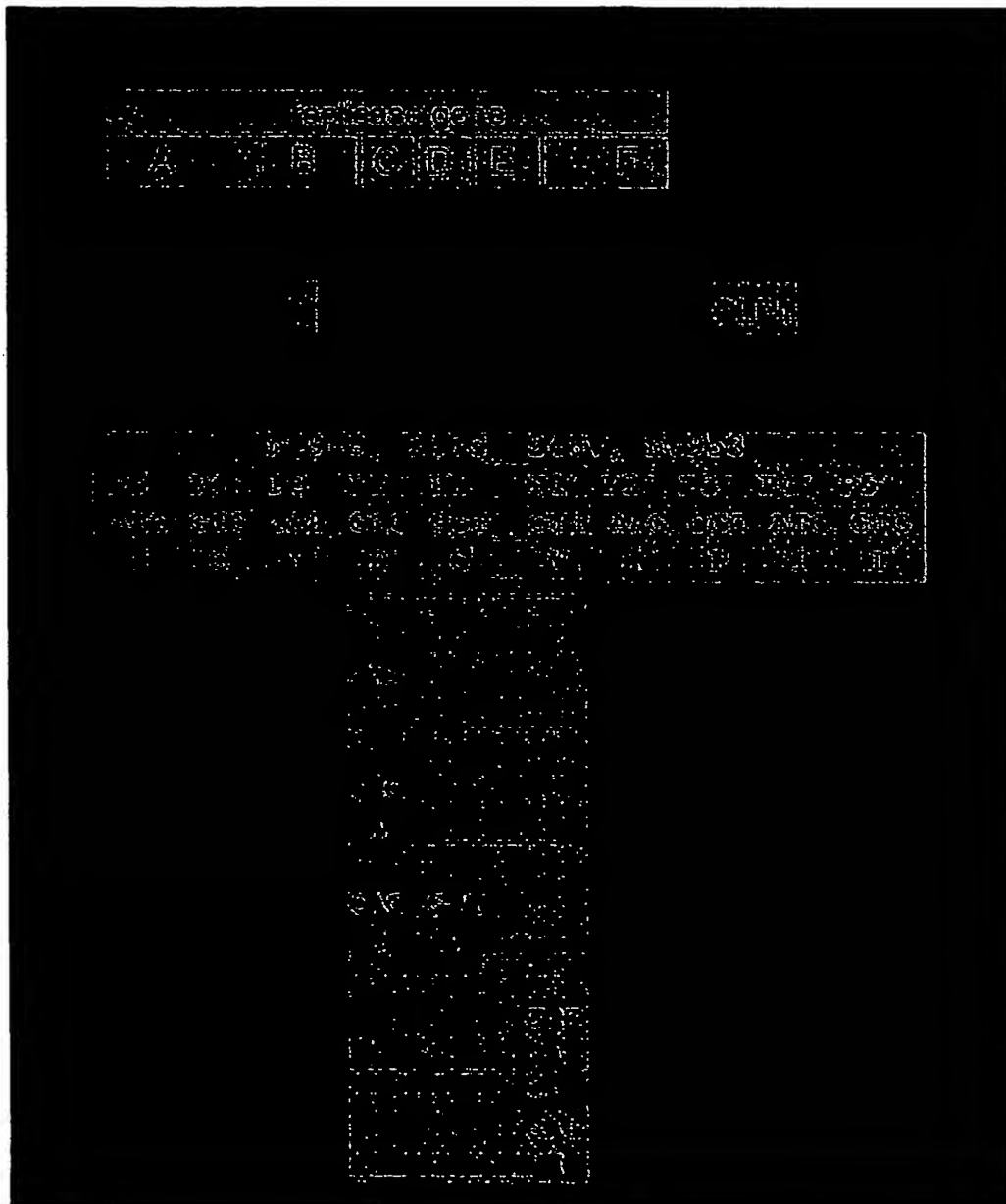


FIG. 4

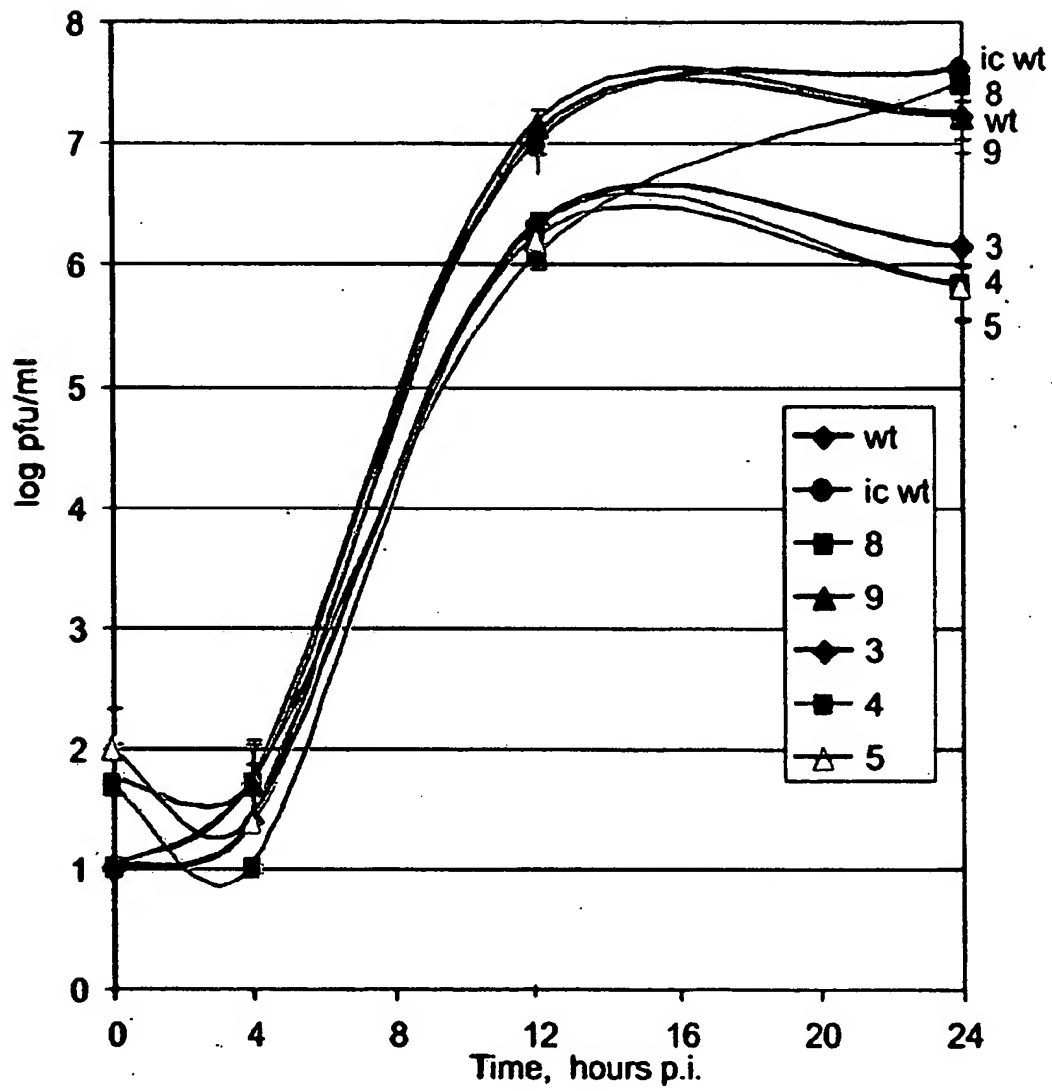


FIG. 5

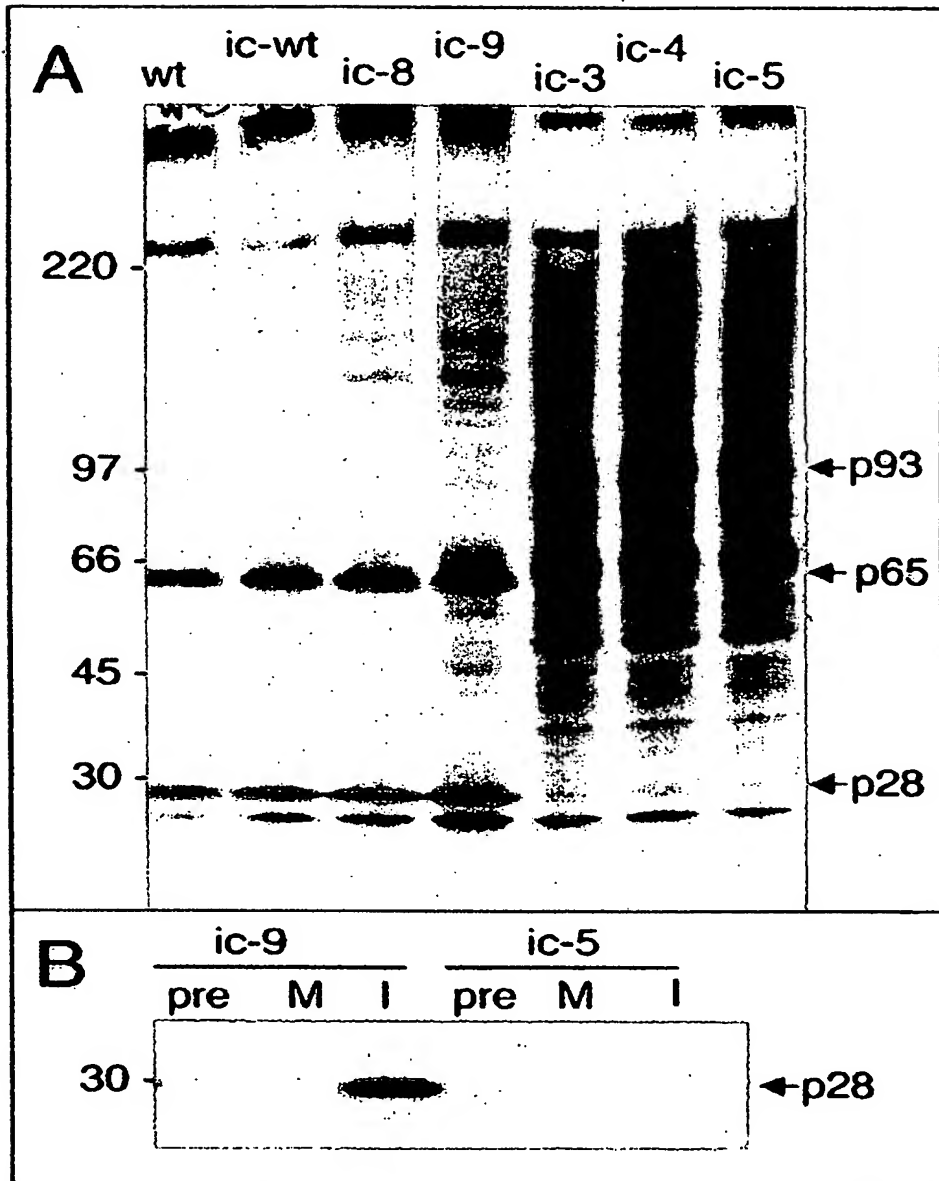


FIG. 6

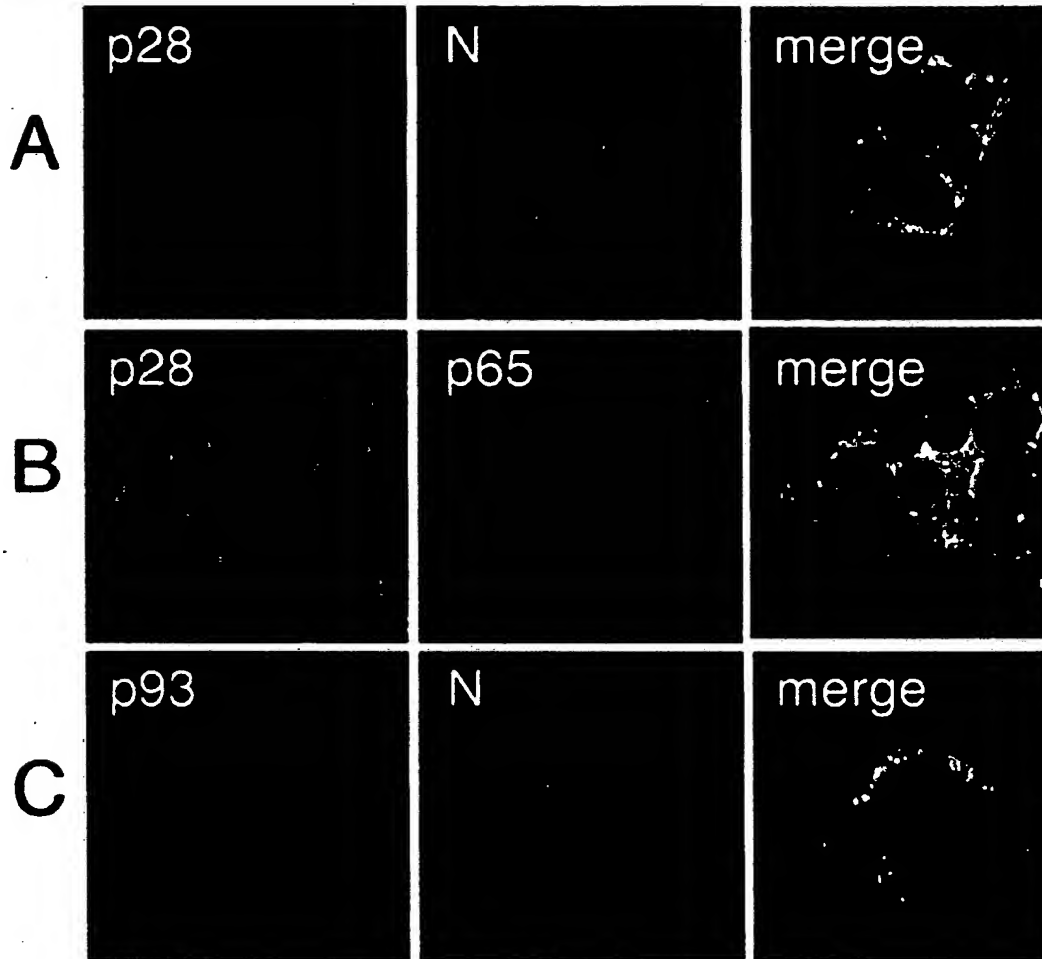
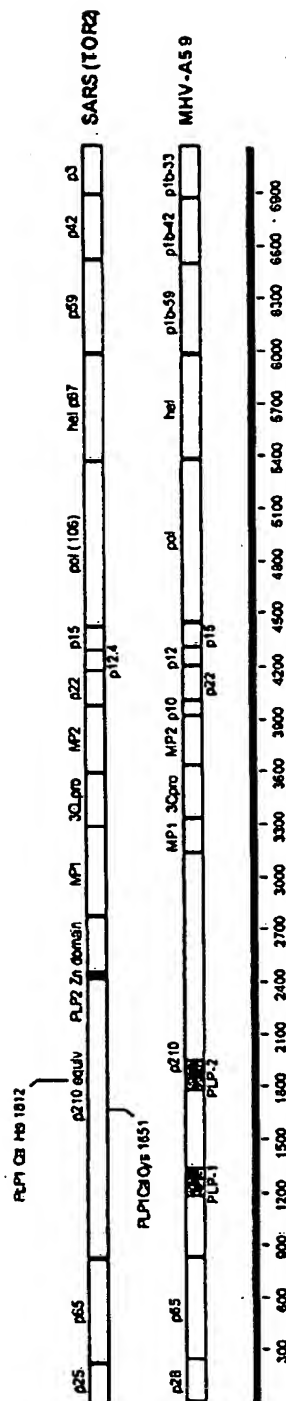


FIG. 7



**FIG. 8**

CS1	P5	P4	P3	P2	P1	P1'	P2'
Wildtype	Lys 243	Gly 244	Tyr 245	Arg 246	Gly 247	Val 248	Lys 249
Knockout	Ile			**His **Ala **His *	*Ala (3) *Val (4) *Val (5)		
Cleaving	Lys	Ala, His Tyr	*Ser *Ala			**Ala (8) *His (9)	*Ala

CS2	P5	P4	P3	P2	P1	P1'	P2'
Wildtype	Arg 828	Phe 829	Pro 830	Cys 831	Ala 832	Gly 833	Lys 834
Knockout	Thr			Gly		Val	
Cleaving	Lys	Cys,	Ala, Thr	Ser	Gly	Ala	Arg

FIG. 9

# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US04/012441

International filing date: 22 April 2004 (22.04.2004)

Document type: Certified copy of priority document

Document details: Country/Office: US  
Number: 60/464,456  
Filing date: 23 April 2003 (23.04.2003)

Date of receipt at the International Bureau: 22 November 2004 (22.11.2004)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

This Page is inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ BLACK BORDERS
- ☒ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☒ COLORED OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images problems checked, please do not report the problems to the IFW Image Problem Mailbox**